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Spermatogonial stem cells: What does the future hold?

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Abstract

Spermatogonial stem cells (SSCs) are responsible for the preservation of spermatogenesis throughout a man's adult reproductive life. Like other stem cells in the body, SSCs can either self-renew or differentiate.
Recent research has shown that SSCs can be considered as multipotent stem cells which can differentiate into cell types other than exclusively gametes. SSC proliferation is a well regulated mechanism and is mainly orchestrated by the Sertoli cells. In the future, SSCs may offer clinically relevant options for preservation and re-establishment of the reproductive potential in patients suffering from spermatogonial stem cell loss, i.e. after gonadotoxic treatments for cancer or haematological diseases.
However, one day, differentiation in-vitro of SSCs may also become an important strategy in other fields of regenerative medicine.

Key words: Testis, stem cell, spermatogenesis, fertility preservation, regenerative medicine.

Spermatogenesis occurs from puberty onwards throughout a man's entire adult reproductive life. The cells responsible for the preservation of this process are the spermatogonial stem cells (SSCs). SSCs derive from the growth-arrested gonocytes of the newborn testis and are situated at the periphery of the seminiferous tubules. Like other stem cells in the body, SSCs can either self-renew to maintain the SSC population or differentiate and give rise to cells from the germ cell line. The proliferation is a well regulated mechanism, which is mainly directed by the Sertoli cells, their direct niche cells.

Spermatogonial stem cell transplantation: a historical perspective

The introduction of spermatogonial stem cell transplantation (SSCT) in the mouse by Brinster *et al.* in 1994 facilitated research on the basic biology of SSCs, but also opened new prospects for fertility preservation in young cancer patients (Brinster and Zimmerman, 1994). This technique involves the injection of a testicular cell suspension from a fertile donor into the testis of an infertile recipient. Spermatogonial stem cells are able to relocate onto the basement membrane and colonize the tubules during the first month after transplantation. From that moment, SSCs start to proliferate and initiate spermatogenesis (Nagano *et al.*, 1998). In a mouse model, research was carried out in our research group BITE (biology of the testis) to test the efficiency and safety of SSCT after in-vivo fertilization, IVF and ICSI. These studies revealed that fertility could be re-established in an otherwise sterile recipient (Goossens *et al.*, 2003) and that healthy offspring were born after SSCT between syngenic individuals. The offspring carried normal karyotypes (Goossens *et al.*, 2010) and presented typical imprinting patterns (Goossens *et al.*, 2009).

SSCT as a means to preserve fertility

The survival of children diagnosed with cancer or haematological diseases (e.g. Sickle cell anaemia, thalassemia) has improved thanks to more effective radio- and chemotherapy. These treatments will

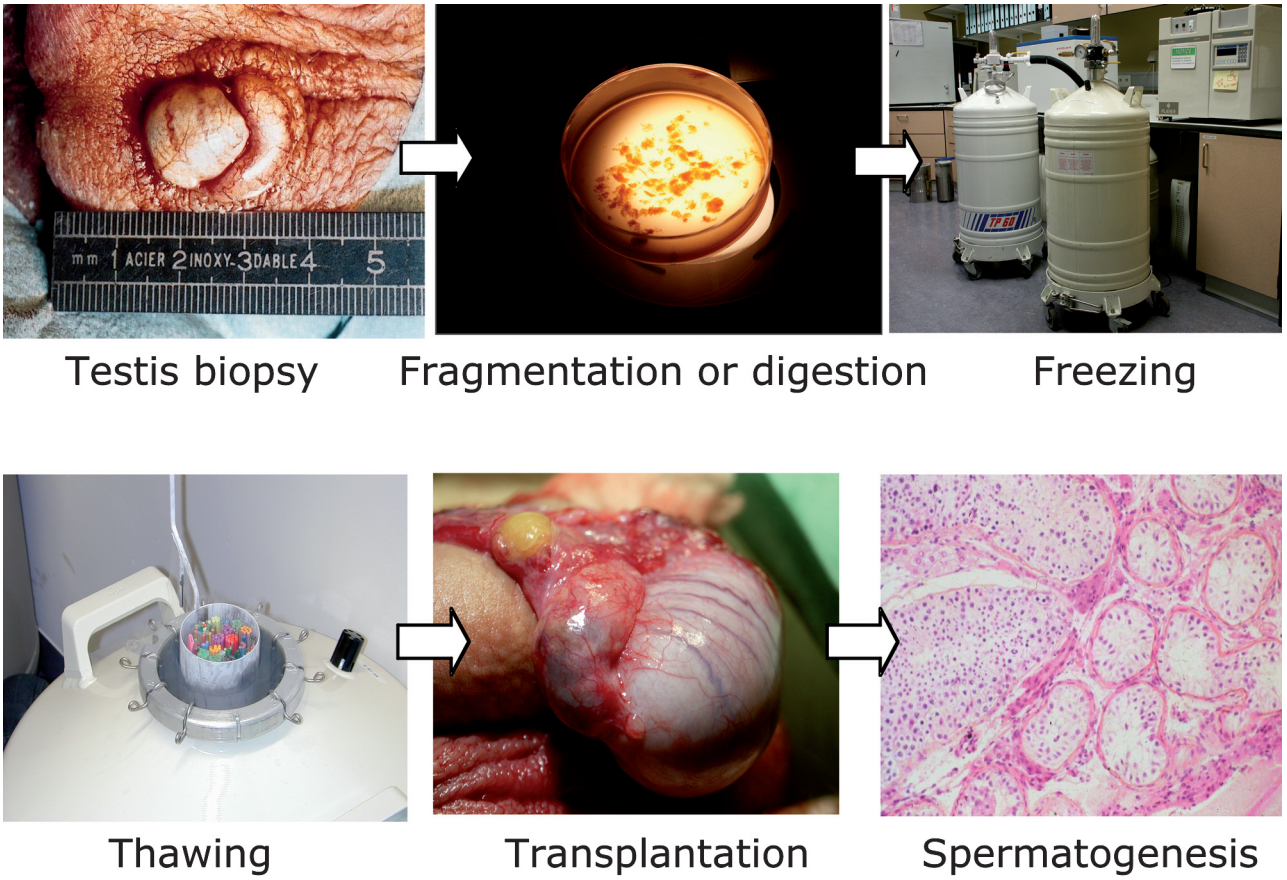

Fig. 1. — Spermatogonial stem cell transplantation as a potential clinical application. Testicular tissue is removed and cryopreserved as tissue or as a cell suspension before the onset of the cancer treatment. After the patient has been cured, the thawed tissue or cells can be transplanted into the remaining testis. When the boy reaches puberty, spermatogenesis may be established.

indiscriminately destroy diseased as well as healthy cells. As a result, SSC failure and infertility may occur. Since 1‰ of the adults in the age group of 20–30 years old is a childhood cancer survivor (Hawkins and Stevens, 1996), prevention of sterility becomes an important goal in reproductive medicine. Since spermatogenesis only starts around puberty, prepubertal boys cannot benefit from the possibility to cryopreserve semen before the onset of their treatment. Cryopreservation of testicular tissue followed by SSCT may offer new strategies for the preservation of fertility in young pre-pubertal boys that do not show active spermatogenesis (Tournaye *et al.*, 1994) (Fig. 1).

Moreover, SSCT can be valuable for adult men too. Cancer patients, who start mild cancer treatments, are not always referred to the fertility clinic. However, when these patients did not recover from the disease and need a more aggressive cure in a later stage, semen parameters are often too bad for sperm cryopreservation. The banking of testicular tissue might be the only way to preserve the fertility in these patients.

The cryopreservation of SSCs is an important tool for fertility preservation. In 1996, Avarbock and co-workers were the first to report on successful cryopreservation of SSCs (Avarbock *et al.*, 1996). Although frozen-thawed suspensions of mouse SSCs were able to colonize recipient testes and initiate spermatogenesis, the search for an optimal freezing protocol is still ongoing. In 2002, a non-controlled-rate freezing protocol was described to yield the highest number (70%) of surviving bovine testicular cells after freeze-thawing (Izadyar *et al.*, 2002). Interestingly, the survival rate of SSCs was higher compared with other testicular cells, which resulted in an enrichment of stem cells in the final suspension. In humans, post-thaw viability of up to 60% was achieved (Brook *et al.*, 2001).

An alternative way of preserving SSCs is to cryopreserve testicular tissue. This method has the advantage that the stem cell niche, important for SSC survival and maturation, is maintained. We developed a non-controlled rate freezing method for prepubertal mouse testicular tissue based on the protocol for SSC suspensions (Goossens *et al.*,

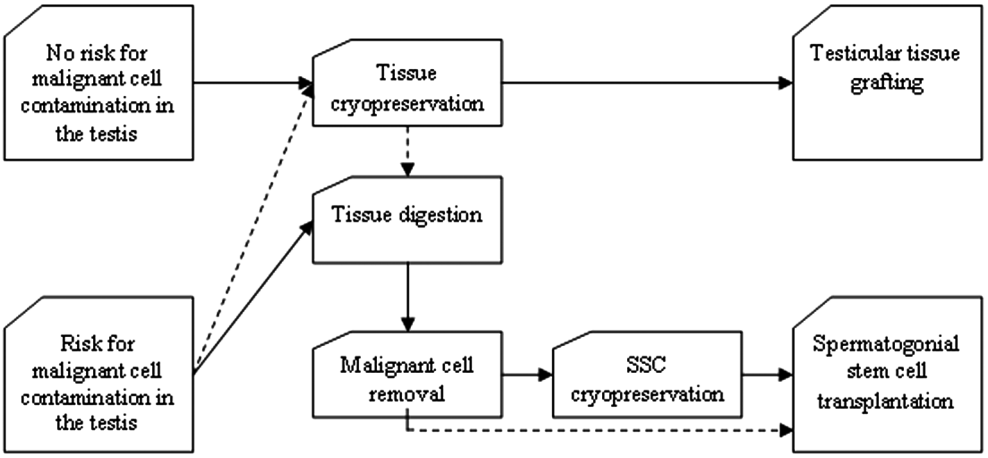

Fig. 2. — Fertility preservation and restoration strategies depend on the malignancy of the disease. When there is no risk for contaminating malignant cells in the testis, tissue preservation and transplantation is the method of choice. In case the risk for contaminating cells in the testis is substantial, the tissue should be digested and decontaminated before (solid arrow) or after (dashed arrow) cryopreservation.

Table 1. — Expression of spermatogonial stem cell markers.

Marker	Selection method
SSEA-4	Positive selection SSC
Thy1	Positive selection SSC
α6-integrin	Positive selection SSC
β1-integrin	Positive selection SSC
CD9	Positive selection SSC
GFRα1	Positive selection SSC
c-ret	Positive selection SSC
c-kit	Negative selection SSC
MHC-I	Positive selection cancer cells
	Negative selection SSC

2008). Although frozen-thawed testicular tissue was able to initiate spermatogenesis, too many SSCs did not survive or lost their function after freezing and thawing. Recently, different protocols for controlled freezing of human prepubertal tissue have been proposed (Kvist *et al.*, 2006; Keros *et al.*, 2007; Wyns *et al.*, 2008). While the integrity of the tissue could be preserved, functional evaluations could not be realized. However, we reported that, even if the SSC survival rate is high, an important loss of functional SSCs can be observed after freezing (Frederickx *et al.*, 2004).

Since it is of high importance that the function of SSCs is maintained during the freezing and thawing procedure, more studies should be conducted paying attention to the functional capacities of human prepubertal SSCs.

Fertility preservation: what route to take?

Which fertility preservation strategy has to be chosen, depends on the malignancy of the disease (Fig. 2). When there is a risk for contaminating cells in the testis, the tissue should be digested and decontaminated before or after cryopreservation. Elimination of cancer cells can be achieved by decontaminating the testis sample using magnetic activated cell sorting (MACS) or fluorescent activating cell sorting (FACS) to select out cancer cells and/or positively select spermatogonia. It was reported in mice that malignant contamination could be overcome by depleting the cell suspension from leukemic cells by FACS prior to transplantation (Fujita *et al.*, 2005). However, we were not able to remove all contaminating cells, using similar decontamination strategies for human testicular cell suspensions (Geens *et al.*, 2007). Other strategies, such as cell sorting for CD49f in combination with differential plating and culture also showed to be inefficient (Geens *et al.*, 2010). It would be worthwhile to improve these decontamination strategies by using additional or more specific markers for SSCs (Table 1).

When there is no risk for contaminating malignant cells in the testis (eg. benign haematopoietic disorders or solid non-metastasing tumours), tissue preservation and testicular tissue grafting would be the method of choice. Attempts have been made to graft immature and adult testicular tissue in both ectopic and homotopic locations. In all studies of mouse-to-mouse ectopic grafts, mouse testicular tissue derived from newborn mice completed spermatogenesis. Grafting frozen-thawed testicular tissue was also found to be efficient (Reis *et al.*, 2000; Honaramooz, 2002). However, results

obtained from adult tissue grafting were less promising (Schlatt *et al.*, 2002). Better results were obtained by our research group after transplanting human prepubertal tissue intratesticularly. Nine months afters transplantation, we could show the survival of SSCs and the differentiation up to the level of secondary spermatocytes (Van Saen *et al.*, 2010). This long-term survival and capacity to initiate differentiation demonstrates the applicability of testicular tissue grafting as a fertility preservation strategy.

Because biopsies obtained from young boys are small and might contain too few SSC for an efficient recolonisation after transplantation, the multiplication of SSC in-vitro would be of great value. SSC culture could be carried out before or after cryopreservation. In case of a short time interval between the biopsy and the transplantation, culture could even be an alternative to cryopreservation. Kanatsu-Shinohara *et al.* reported a technique for culturing SSCs for at least six months in the absence of both serum and feeder cells (Kanatsu-Shinohara *et al.*, 2010). Although the cultured cells completed spermatogenesis and produced offspring following SSCT, further investigations on the efficiency and safety of SSC culture is warranted.

Spermatogenesis in-vitro and beyond

In addition, in-vitro culture of SSC could be the initial step to study in-vitro differentiation and maturation with the aim of establishing spermatogenesis in-vitro. Culture and differentiation of male germ cells has been performed for various purposes in the past, but none of the studies has resulted in a sufficient number of mature gametes. Stukenborg *et al.* demonstrated that morphologically normal spermatozoa could be obtained from immature mouse germ cells through 3D culture. Further research is required to reveal the applicability of this culture technique for human germ cells and the functionality of the spermatozoa by generating offspring (Stukenborg *et al.*, 2009).

The culture system as well as SSCT together with transfection of SSCs may prove very useful methods in achieving more knowledge of SSC biology and function. Studies, using these methods, may reveal more insight in male infertility. Infertility occurs in 13-18% of the couples seeking to have children. In 50% of these couples, the cause of infertility is of paternal origin. Some specific male-factor disorders can be treated, but for non-specific male-factor infertility, treatment is difficult. In order to recognize, and eventually treat these defects, it is critical to identify the regulating mechanisms at the various stages of spermatogenesis.

By culturing spermatogonia in-vitro, more knowledge on spermatogenesis regulation may also lead to novel strategies in male contraception. There are several approaches to male contraception, but research and development of male hormonal contraceptives is the only one at the stage of clinical research. Hormonal contraceptives, however, have some adverse effects such as modest bodyweight gain and suppression of high density lipoprotein-cholesterol levels.

Studies on SSCs can also prove useful for the treatment of germinal tumours. Testicular cancers are the most frequent tumour of the young adult (20–35 years). The current understanding is that tumours progress from a carcinoma in situ, which in turn are malignant cells derived from gonocytes (Jorgensen *et al.*, 1990). Because, at this moment, there is no animal model representing the characteristics of the human germinal tumours, studies using SSC culture will be of great significance.

SSCs and regenerative medicine

In the adult mouse, SSCs were reported to show pluripotent characteristics after *in-vitro* culture. SSCs were shown to spontaneously differentiate *in-vitro* into cell lines of all embryonic germ layers and to form teratomas when injected to immunodeficient mice (Guan *et al.*, 2006). Later it was reported that also adult human SSCs show pluripotent characteristics (Conrad *et al.*, 2008; Kossack *et al.*, 2009; Golestaneh *et al.*, 2009; Mizrak *et al.*, 2009). Adult SSCs can thus eventually become a source of pluripotent cells that can be differentiated into cells from different lineages for regenerative purposes. When more studies corroborate their true pluripotent nature and safety in transplantation experiments, they can become a valuable alternative to human embryonic stem cells. Although they are more acceptable from an ethical viewpoint and they may allow to derive patient-specific cell lineages, they can eventually only be generated and used in male patients.

Summary

In conclusion, we believe that SSCs may offer clinically relevant options for preservation and re-establishment of the reproductive potential in patients suffering from SSC loss. We consider testicular tissue grafting as the first choice for patients with benign diseases or solid tumours, while SSC transplantation will be proposed for patients who are at risk of having malignant cells in their testes. One day, differentiation in-vitro of SSCs may become an important strategy in patients with a spermatogenic arrest because of deficiencies at the level of the niche cells, but even maybe also in other fields of regenerative medicine.

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